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Characterization of *Leuconostoc oenos* Isolated from Oregon Wines†

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This study was designed to characterize isolates of *Leuconostoc* species from Oregon wines. Gram-positive cocci were isolated, and their biochemical properties and abilities to decompose malic acid were determined. All of the isolates were heterofermentative, catalase negative, and facultatively anaerobic and occurred in pairs and chains. They produced acid from glucose, fructose, mannose, ribose, cellobiose, trehalose, and salicin but not from sucrose or lactose. They did not produce ammonia from arginine or dextran from sucrose. They grew at pH values of less than 4 and in 10% ethanol. Most but not all strains produced lactic acid and carbon dioxide from malic acid, as determined by paper chromatography and respirometry, respectively. These malolactic bacteria were considered to be strains of *Leuconostoc oenos*. We compared these isolates with reference strains for relative growth at pH values of 4.0, 3.5, 3.0, and 2.8 at 22°C. The isolates were similar in their growth responses at the two highest pH levels. At pH 3.0 and 2.8, however, the strains failed to grow but revealed variable abilities to dissimilate malic acid.

The malolactic bacteria are lactic acid bacteria which carry out a secondary fermentation during storage of new wine (2, 11). The following three genera of lactic acid bacteria have been implicated in this phenomenon: *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (9, 11, 20). The members of one of these genera, *Leuconostoc* spp., have been found to carry out the fermentation most frequently and have been found to be most desirable (9, 11, 20). This fermentation, called malolactic fermentation (MLF), is especially important in cold climatic regions (3, 11), such as Oregon, where the wines are more acidic, necessitating the decarboxylation of malic acid (a dicarboxylic acid) to lactic acid (a monocarboxylic acid). It is also important in warm climates where the pH of the wines is so high that it is difficult to prevent the fermentation (2, 11, 17, 19, 25). MLF generally manifests itself as an effervescence from escaping carbon dioxide, an increase in turbidity due to bacterial growth, and a slight decrease in the color of red wines (11).

Apart from decreasing the acidity of wine, MLF has two other important effects. One of these is bacteriological stability, and if MLF occurs during or after the primary yeast alcoholic fermentation of the juice and subsequent storage, the malolactic bacteria present in the wine after bottling carry out the secondary fermentation during warehousing and distribution. This leads to haze and sediment formation in the bottle, as well as an increase in pH and a build-up of carbon dioxide (11, 20). Therefore, the wine may not be acceptable to consumers on the basis of appearance. A second and more subtle effect is the change in flavor due to elevated amounts of bacterial end products, such as aldehydes, esters, and diacetyl, which, at threshold levels, contribute to the bouquet of wine (2, 8, 11).

The MLF is catalyzed by the malolactic enzyme which requires NAD^+ and Mn^{2+} in producing L-lactate and CO_2 from L-malate. This enzyme has been purified from *Leuconostoc mesenteroides* (13) and from *Leuconostoc oenos* (23) and has been cloned in *Escherichia coli* and *Saccharomyces cerevisiae* (24).

Grapes grown in cool climates usually are lower in sugar and higher in acid than grapes grown in warmer climates, and for the former this may result in the production of wines with harsh tastes. For example, the titratable acidities and sugar contents of Oregon musts range from 0.93 to 1.62 g of tartaric acid per 100 ml and from 15.7 to 22.6° Brix, respectively; the titratable acidities and sugar contents of California musts range from 0.5 to 1.0 g of tartaric acid per 100 ml and from 20 to 25° Brix, respectively (22, 25). This difference may be due to the climate, soil, grape varieties, and fermentation procedures, all factors known to influence the quality of wines produced in various places (21). As a result, the MLF is desirable to reduce the acidity of certain wines. However, the occurrence of the fermentation is variable, which may be due to low temperatures and pH values.

Pure culture inoculation with *L. oenos* ML-34, which is used commercially in California and other places (15, 20), is practiced to some extent in Oregon, but this organism is not well-suited to the low temperature and pH conditions prevalent during wine production in the Northwest. The possibility of using malolactic bacteria indigenous to Oregon wines to reduce wine acidity initiated this study. Our objectives included isolation of *L. oenos* from wines active in carrying out the MLF, characterization of such bacteria to confirm their identity and ability to decompose malate, and selection of the most active strains for possible pure culture inoculation to improve wine quality. Since it has been shown that the wine *Leuconostoc* strains are better adapted to hostile wine environments and are more effective in carrying out the MLF (11, 17), we concentrated on isolating and

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characterizing such malolactic bacteria. Isolates which were lactobacilli or pediococci were not studied.

MATERIALS AND METHODS

Sources of bacteria. Several samples of 1978 Pinot Noir, Chardonnay, and Merlot varieties obtained from the McMinnville and Dundee wineries in Oregon were used as sources of the bacteria isolated from wine. Stock control strains of *L. oenos* were obtained from R. E. Kunkee, University of California at Davis (strain ML-34), and from Tri-Bio Laboratories, State College, Pa. (strain PSU-1).

Media and isolation. Tomato juice-glucose-fructose-malate broth (TGFMB), a modified Rogosa medium (8, 16) containing 2% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% Bacto-Peptone (Difco), 0.5% glucose, 0.3% fructose, 0.2% malate, 2 drops of Tween 80 (Difco) per liter, and 325 ml of yeast extract per liter in 4.2-fold-diluted tomato juice (canned without preservatives) was used for isolation. The diluted tomato juice was filtered through analytical filter paper (Schleicher & Schuell Inc., Keene, N.H.) and Whatman glass microfiber filter paper with the aid of diatomaceous earth (Sigma Chemical Co., St. Louis, Mo.). To avoid haze and sediment formation in the medium, the yeast extract was prepared by blending commercial compressed bakers' yeast (Fleischman's; Standard Brands, Inc., New York, N.Y.) at low speed with sufficient distilled water to form a 20% suspension, which was autoclaved for 30 min at 121°C. The solids were allowed to settle overnight at 4°C, and the cloudy supernatant was clarified by centrifugation. The resulting clear solution was frozen and stored at −20°C. The pH of the medium was adjusted to 5.5 with potassium hydroxide. Basal broth lacked tomato juice, glucose, and fructose. Tomato-glucose broth lacked fructose.

The bacteria were isolated from the wine samples by making pour plates containing 0.1-ml diluted wine samples in TGFMB agar supplemented with 50 mg of cycloheximide (Sigma) per liter to inhibit yeast growth. Characteristic lactic acid bacterial colonies were picked and examined microscopically to insure that they were gram-positive cocci in pairs and chains. Representatives of such colonies were cultured in TGFMB and maintained in stab cultures of the same agar at 4°C. The cultures were incubated in a GasPak anaerobic system (BBL Microbiology Systems, Cockeysville, Md.) at 30°C for 3 to 4 days before refrigeration. A total of 23 strains were isolated in this way.

Characterization of bacteria. Isolates were characterized in comparison with *L. oenos* ML-34 and PSU-1. Catalase activity, dextran production from sucrose, ammonia production from arginine, and production of lactic acid from glucose were determined as previously described (5, 15). The isomeric form of lactic acid produced from glucose was determined by colorimetric measurement of the total lactic acid (16) and enzymatic measurement of L-(+)-lactate (7).

(i) Utilization of organic acids. The ability to utilize L-malate and the ability to utilize citrate were determined by growing the isolates in tomato-glucose broth containing 0.2% L-malate or 0.2% citrate. Uninoculated media were used as controls. The presence or absence of the acids was determined after growth for 7 days by using paper chromatography, a butanol-water-formic acid (1:1:0.17) solvent system, and bromcresol green as a dye (10, 11).

(ii) Effect of added malate on growth. The effects of different levels of malate on the growth of isolates were determined by adding 0.05, 0.10, 0.15, 0.20, and 0.25% malate to tomato-glucose broth (pH 5.5) and making turbidity readings with a Bausch & Lomb Spectronic 20 spectrophotometer at 600 nm after growth for 3 days at 30°C.

(iii) Energy sources. Carbohydrate utilization was determined by (i) growing the isolates at 30°C in autoclaved basal broth containing the membrane-filtered sugar to be tested at a concentration of 0.5%, (ii) the Minitek differentiation system (BBL), in which 0.05-ml portions of washed cells (see below) were added to the various sugar disks incubated at 30°C in the GasPak anaerobic system, and (iii) growing the isolates in a synthetic medium as previously described (5). Uninoculated media were used as controls.

(iv) Effect of pH on growth and malate dissimilation. The ability of the isolates to grow and the ability of the isolates to ferment malic acid at low pH values were tested at pH 2.8, 3.0, 3.5, and 4.0. Incubation was in sterile (121°C, 15 min) TGFMB for 24 days at 22°C. Growth also was determined at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5 and in presence of 10% ethanol. The pH was adjusted with hydrochloric acid or potassium hydroxide by using a Corning model 125 digital pH meter.

(v) Cellular dry weight determination. The dry weights of the cells were compared by growing the isolates in 100-ml portions of TGFMB (pH 5.5) for 7 days at 30°C, after which the cells were centrifuged at 10,400 × *g* for 20 min. The cells were washed with an equal volume of potassium phosphate buffer (pH 5.5), centrifuged, and suspended in an equal volume of the buffer. The washed cells were again centrifuged, and the resulting pellet was suspended in 15 ml of distilled water; 5 ml of the suspension was then pipetted into a preweighed beaker and dried in an oven at 60°C overnight. Sufficient buffer was then added to the remaining 10 ml, which was centrifuged, and the pellet was used after suspension in 10 ml of buffer for detection of carbon dioxide production from malate by respirometry as described below.

Detection of malolactic activity. (i) Paper chromatography and enzymatic methods. Since personnel in wineries use the chromatographic spot test for wine, this procedure was followed, using the butanol-water-formic acid solvent system described above. Spots were made from the supernatants of centrifuged TGFMB cultures after growth for 7 days at 30°C. Malate and lactate (0.2% each) were used as standards, and uninoculated broth and *L. oenos* ML-34 cultures were used as controls. Disappearance of the malate spot was indicative of positive fermentation (11). The relative malate-utilizing abilities of different isolates and control strains were tested in a grape juice medium containing 0.5% Difco Proteose Peptone, 0.5% tryptone (Difco), 0.5% yeast extract (Yeast Products Inc.), 0.2% L-malic acid (Sigma), 0.2% ammonium citrate (dibasic; J. T. Baker Chemical Co., Phillipsburg, N.J.), 0.5% sodium acetate (Mallinckrodt), 0.1% Tween 80 (Baker), 0.5% potassium phosphate (dibasic; Mallinckrodt), 0.05% $MgSO_4 \cdot 7H_2O$ (Mallinckrodt), 0.02% $MnCl_2 \cdot 4H_2O$ (Mallinckrodt), and 0.005% $FeSO_4 \cdot 4H_2O$ (Mallinckrodt) dissolved in 100 ml of white grape juice (Welches). The pH was adjusted to 5.5 with 6 N NaOH. Flasks containing 100 ml of the medium were inoculated with 2.0 ml of the desired strain previously grown for 5 days in TGFMB and adjusted to an optical density at 600 nm of 0.7. Incubation was at 22°C for 7 days, and the average rate of malate fermentation per day was calculated from daily enzymatic determinations of the malic acid content of each flask. The method of McCloskey (14) was used to assay for malic acid.

(ii) Respirometry. An eight-channel differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.) was used to determine carbon dioxide production from

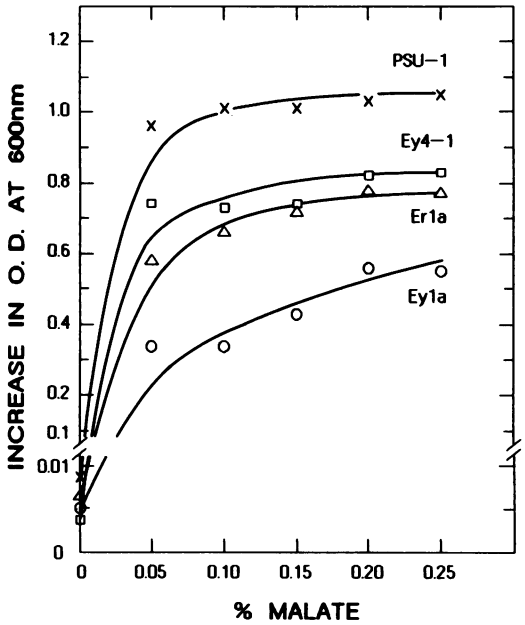

FIG. 1. Influence of added malic acid on optical density reached in 3 days at 30°C by three of our isolates and *L. oenos* PSU-1. O.D., Optical density.

malate as previously described (12), except that 1 ml of washed cells (see above) was added to the side arm of the 15-ml reaction vessel instead of cell-free extract preparations. Gas evolution was measured after mixing at 10-min intervals for 60 to 90 min. To compare the relative malate decomposing abilities of the isolates and reference strains, the amount of carbon dioxide produced per milligram of cell dry weight was determined.

RESULTS

Of the 23 isolates obtained, 12 were fully characterized and compared for their ability to decompose malate. Because of the fastidious nature of wine *Leuconostoc* isolates (9), the medium containing tomato juice was required for reasonable growth. It also has been noted by other workers that these organisms grow best in media containing this and other complex nutrients (1, 8, 9, 19, 26). We also found that they grew best when they were incubated anaerobically in the GasPak system.

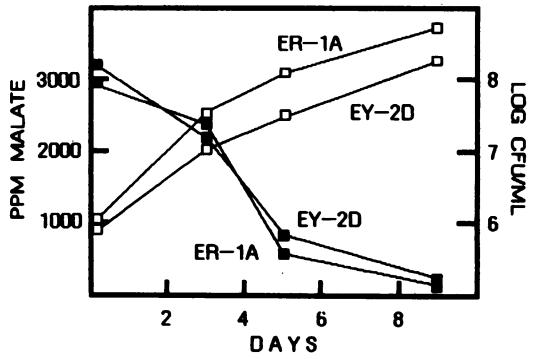

FIG. 3. Malate fermentation (■) and growth (□) at pH 3.5 for *L. oenos* Er1a and Ey2d at 22°C.

Characteristics. All of the isolates were gram-positive cocci, occurred in pairs and chains, were facultatively anaerobic, catalase negative, and heterofermentative, and did not produce ammonia from L-arginine or dextran from sucrose; they did produce lactic acid from carbohydrates and gas from glucose. They utilized glucose, fructose, ribose, trehalose, mannose, cellobiose, and salicin. Maltose, galactose, raffinose, esculin, and arabinose were utilized by some strains, but sucrose, xylose, and lactose were not utilized by any of the isolates as energy sources. The organisms utilized L-malate and citrate in the presence of glucose, from which they produced D-(−)-lactate. They grew in 10% ethanol and at pH 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, and 3.5. These characters are similar to those of *L. oenos* published in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (4), and elsewhere (5, 15).

Effect of added malate on growth. Figure 1 shows the effect of malate on the growth of some of the isolates. *L. oenos* PSU-1 responded best, and 0.2% malate caused a maximum response for all isolates. By 3 days, all of the strains reached the maximum stationary phase at 30°C, when the optimum level of malate was present.

Effect of pH on growth and malate dissimilation. Figures 2 and 3 show the fermentation rates at pH 3.5 for reference strains ML-34 and PSU-1, as well as isolates Er1a and Ey2d. Figures 4 and 5 show data for the same strains at pH 3.0. The data at pH 2.8, like the data at pH 3.0, where the cells did not grow, and at pH 4.0, where the cells grew rapidly, are not shown. Except for strain ML-34, the isolates appeared similar in their growth responses at the higher pH levels (pH 4.0 and 3.5). At 20°C and pH 4.0 and 3.5, strain ML-34

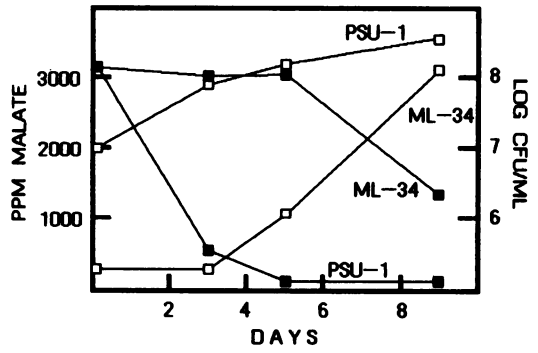

FIG. 2. Malate fermentation (■) and growth (□) at pH 3.5 for *L. oenos* ML-34 and PSU-1 at 22°C.

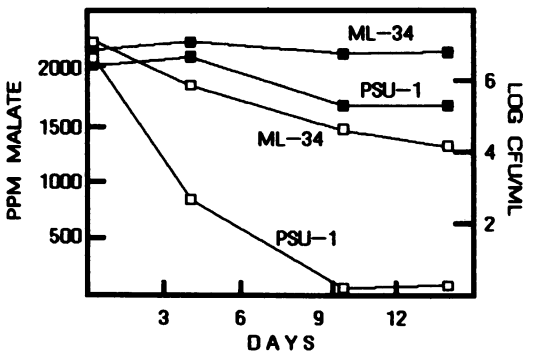

FIG. 4. Malate fermentation (■) and growth (□) at pH 3.0 for *L. oenos* ML-34 and PSU-1 at 22°C.

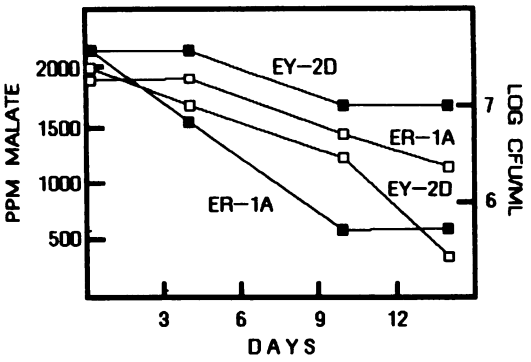

FIG. 5. Malate fermentation (■) and growth (□) at pH 3.0 for *L. oenos* Erla and Ey2d at 22°C.

showed an increase in turbidity compared with the Oregon wine isolates.

At 30°C, the isolates grew best at pH 5.5; hence, this pH value was chosen for laboratory experiments. Furthermore, the isolates were capable of growth in media containing 10% ethanol. The capacity to grow at pH values less than 5 and the capacity to grow in the presence of 10% ethanol are characteristic of *L. oenos* (5).

Cellular dry weight and malolactic activity. Table 1 shows a comparison of the cellular weights of our isolates and reference strains ML-34 and PSU-1 in relation to their malate-decomposing abilities at 30°C. The dry weights ranged from 0.12 to 2.1 mg/ml, and this seemed to influence the relative malolactic activities of the isolates, as evidenced by the variable responses of strains Erla, Er3b, and Ey4-2b determined by paper chromatography. It is evident that some of the isolates grew better and produced more CO_2 per minute than strains ML-34 and PSU-1. When malate was assayed enzymatically, several isolates, especially strains Erla and Ey2d, were more active than reference strains at 22°C (Table 2).

To confirm that some of the lactic acid produced in the broth was from malic acid and that not all of the lactic acid was from the endogenous carbohydrates in the medium (15), the reaction mixture was chromatographed at the end of each respirometric analysis in which malate was the sole substrate with NAD^+ and Mn^{2+} as cofactors. The results were similar to those shown in Table 1 for MLF. Most of the malate was converted to lactate.

DISCUSSION

Malolactic bacteria have been characterized by several workers, and *L. oenos* has received the greatest attention (3, 5, 6, 8, 15). The characteristics of the isolates described in this paper are similar to those of *L. oenos* published in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (4), and elsewhere (5). They also agree with the results obtained for *L. oenos* ML-34 and *L. oenos* PSU-1 published previously (3, 15).

The failure of our strains to ferment lactose and sucrose separates them from the milk-curdling and slime-forming *Leuconostoc* isolates, respectively. Although sugar utilization has been regarded as unsatisfactory for separation of *L. oenos* from the other *Leuconostoc* species (6), due to residue carbohydrates in complex media (15), the Minitek differentiation system without any carbohydrate other than the test sugar provided reliable results. Furthermore, the isolates did not ferment xylose, whereas arabinose was fermented by some. Previously, *L. oenos* was found to be unable to ferment xylose (3, 15), whereas some strains fermented arabinose (4, 5).

It was shown recently (6) that two of five *L. oenos* strains studied produced ammonia from arginine, which is not characteristic of *L. oenos* (4). All of the isolates in the present study failed to produce ammonia from arginine, in agreement with previously published work (4, 5, 15). We also found that our strains produced lactate from malate and D-(−)-lactate from glucose, as noted by other workers (16, 19), substantiating that our isolates are strains of *L. oenos*.

Tolerance to low pH values (pH < 4.2) and tolerance to 10% ethanol are characters that are used to separate *L. oenos* from the non-acidophilic *Leuconostoc* species (5, 6); our strains exhibited these properties. In fact, our isolates grew in media with pH values as low as 3.5, which is a further indication that they are strains of *L. oenos*. Although

TABLE 1. Comparison of the cellular dry weights and malolactic activities (CO_2 produced) of *L. oenos* isolates grown for 7 days at 30°C and pH 5.5[a]

Strain	Dry wt (mg/ml)	CO_2 production (μl of CO_2 per min per mg)[b]	MLF[c]
Eyc	0.20	30.5	+
Ey4-2	0.27	22.6	+
Ey2d	0.27	24.0	+
Ey3c	2.1	3.4	+
Eyla	1.44	4.6	+
Ey2a	0.27	28.2	+
Ey4-2a	0.57	8.0	+
Erla	0.15	9.1	±
Er2	1.2	10.9	+
Ey4-1	0.3	28.4	+
Er3b	0.21	1.0	±
Ey4-2b	0.12	4.4	±
ML-34	0.3	24.2	+
PSU-1	0.6	8.1	+

[a] Values are averages of triplicate determinations.
[b] The amount of CO_2 produced was measured by using a respirometer.
[c] MLF was determined by paper chromatography. +, Positive; ±, variable response.

TABLE 2. Relative malate fermentation rates of 18 isolates and two reference strains of *L. oenos* in a grape juice medium (0.2% malate) incubated at 22°C for 7 days

Strain	Amt of malate fermented (μg/ml per day)
PSU-1	395
ML-34	327
Erla	544
Erlb	509
Erlc	530
Erld	230
Erle	194
Er3b	534
Er3c	209
Er3d	159
Er3e	262
Er4a	523
Er4c	533
Er4d	518
Eyla	528
Eylc	452
Ey2a	314
Ey2c	537
Ey2d	547
Ey4b	536

they failed to grow at pH 3.0 and 2.8, they nonetheless demonstrated malolactic activity at these pH values.

Malate and citrate were stimulatory for growth of our strains in the presence of glucose; 0.2% malate was optimum, a level which was found to be stimulatory for these bacteria in a previous study (18). It also was reported that 0.08% malate was best for a linear increase in cell yield for *L. oenos* ML-34 in the absence of glucose (15). Therefore, utilization of malate for growth seems to depend on the amount of glucose present. We also noted (Table 1) that most strains with the highest malolactic activity generated lower cell weights. Since the malolactic enzyme is inducible (13), it may have been diluted for cells growing to higher densities as the malate concentration diminished. Low activity could also be the result of simply less growth (e.g., strain Er3b), perhaps because 30°C was too high a temperature for some of the Oregon strains.

Some of the strains isolated during this study appeared to be more efficient at 22°C in malate fermentation than strain ML-34 or PSU-1 (Table 2) and thus appear to be better candidates for pure culture inoculation of Oregon wines. In view of the more acidic nature of these wines, the ability of the strains tested to grow at low pH and temperature values provided the opportunity to test them on the 1983 vintage wines requiring a MLF. These trials have been satisfactory, and the results will be reported elsewhere.

From the results obtained in this study, we conclude that the Oregon wine isolates are strains of *L. oenos*, some of which may be unusual in their ability to carry out the MLF at low temperature and pH levels.

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